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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/68, G01N 33/574

A1

(11) International Publication Number: **WO 00/47765**

(43) International Publication Date: 17 August 2000 (17.08.00)

(21) International Application Number: PCT/GB00/00313

(22) International Filing Date: 3 February 2000 (03.02.00)

(30) Priority Data:
9902776.5 8 February 1999 (08.02.99) GB

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: MATERIALS AND METHODS RELATING TO THE DETECTION OF A CANCER CELL MARKER

(57) Abstract

The invention relates to materials and methods involved in the identification and use of a novel cancer cell marker. The inventors provide nucleic acid sequence and polypeptide sequence for Microphthalmia-associated transcription factor (Mitf) mRNA splice variants (Mitf(-) and Mitf(+)) and methods for detecting the particular variants in cells such as melanoma cells. The inventors have found that the predominance in normal cells of one splice variant as compared to the other is reversed in cancer cells such as melanoma cells. Therefore, this provides a cancer cell marker which can aid analysis of the cells and can be used for diagnosis or prognosis to assist a physician in determining the severity or likely course of the cancer and/or optimise treatment of it.

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MATERIALS AND METHODS RELATING TO THE DETECTION OF A CANCER CELL MARKER

Field of the invention

The present invention relates to materials and methods involved in the identification and use of a novel cancer cell marker. Particularly, but not exclusively, the present invention provides nucleic acid sequence and polypeptide sequence for Microphthalmia-associated transcription factor (Mitf) mRNA splice variants and methods for detecting said variants in cells such as melanoma cells.

Background to the Invention

The bHLH-LZ factor Mitf (Hodgkinson et al (1993) Cell 74, 395-404; and Moore et al (1995) Trends in Genet. 11, 442-448) plays a crucial role in the development of the melanocyte lineage; mice bearing mutations in the *microphthalmia* gene lack neural crest-derived melanocytes (Stringrímsson et al, (1994) Nat. Genetics 8, 256-263; and Opdecamp et al (1997) Development 124, 2377-2386), and have small eyes due to aberrant formation of the retinal pigment epitheliun (RPE) (Nakayama et al (1998). Mec. Dev. 70, 155-166). In humans, Mitf is mutated in individuals suffering from Waardenburg's syndrome type II which is characterised by varying degrees of abnormal pigmentation and deafness, since melanocytes in the inner ear play an essential role in hearing (Steel and Barkway (1989), Development 107, 453-463). In addition, under some circumstances Mitf can convert fibroblasts to cells expressing melanogenic markers (Tachibana et al (1996) Nature Genetics 14, 50-54). Based on transfection assays, Mitf has been shown to activate the expression of the melanocyte-specific genes *Tyrosinases* and *TRP-1* (Bentley at al (1994) Mol. Cell Biol. 14, 7996-8006; Ganss et al (1994)J. Biol. Chem. 269, 29808-29816; Hemesath et al (1994) Genes Dev. 8, 2770-2780; Yasumoto et al (1994)

Mol. Cell Biol. 14, 8058-8070; Yavuzer et al (1995) Oncogene 10, 123-134) through an evolutionarily conserved 11 bp sequence termed the M box (Lowings et al (1992) Mol. Cell. Biol. 12, 3653-3662). Further, the present inventors recently established that Mitf will recognise the sequences TCATGTGA, TCATGAGN or NCATGTGA (Aksan and Goding (1998) Mol. Cell Biol. 18, 6930-6938).

In addition to being expressed in cells of the melanocyte lineage, Mitf is also found in a number of other cell types, including osteoclasts and mast cells. Thus, in addition to defects in the melanocyte lineage, mice lacking functional Mitf also suffer from osteopetrosis, reduced mast cell and natural killer cell numbers.

In the melanocyte lineage where Mitf expression is driven by the MITF-M promoter, the Mitf mRNA is present in two differentially spliced forms which give rise to Mitf proteins which differ by an internal 6 amino acids (ACIFPT) located N-terminal to the DNA binding domain (Stringrímsson et al, (1994) Nat. Genetics 8, 256-263). The spliced form containing the additional 6 amino acids is termed Mitf (+) and the spliced form with the 6 amino acids absent is termed mitf (-). However, little else is known of the different functions of the Mitf (+) and Mitf (-) proteins.

Summary of the Invention

The present inventors have found that in normal cells, the predominant form of Mitf mRNA is Mitf(+) whereas in certain cancer lines tested, the predominant form present of Mitf mRNA is Mitf (-).

Therefore, in a first aspect, the present invention provides the diagnosis of diseases associated with the differentially spliced forms of Mitf by use of specific binding members such as a) nucleic acid molecules

hybridisable with a nucleic acid specific to the (+) or (-) forms of Mitf mRNA or cDNA; b) substances comprising an antibody domain with specificity for epitopes or sequences characteristic of either the (+) or (-) forms of Mitf nucleic acid or polypeptide.

The nucleic acid binding members may take the form of probes for detecting nucleic acid sequences specific for either (+) or (-) forms. As the Mitf(+) and Mitf(-) forms are splice variants, the genomic nucleic acid sequence will not distinguish between the two forms. However, the expressed mRNA will contain nucleic acid sequence specifically characteristic for both forms respectively. Likewise, the encoded polypeptides will have unique amino acid sequence. These characteristic properties may be utilised in connection with the present invention. The nucleic acid probes preferably comprise sequence having sufficient homology with the distinctive nucleic acid sequences of the Mitf (+) and Mitf (-) forms such that they will hybridize under relatively stringent conditions. The sequence of the probes may be conveniently derived from the sequence shown in Fig. 1, preferably comprising the region of the Mitf (+) as indicated. The probes are preferably at least 10 bp in length, more preferably at least 25bp in length, even more preferably at least 40bp in length and most preferably between 20 and 200bp in length. The sequence of the probe, as derived from the sequence as shown in Fig. 1, preferably has at least 60% homology with the sequence comprising the Mitf(+) sequence, even more preferably 70%, most preferably 85% homology and particularly preferably 95% homology. In a further aspect of the present invention there is provided a nucleic acid sequence having at least 20bp (and preferably no more than 100bp) comprising the sequence as shown in Figure 1 spanning the region indicated as Mitf(+) or its

complement sequence for use detecting the Mitf (+) spliced form of Mitf. In another aspect there is provided a nucleic acid sequence having at least 20bp (and preferably no more than 100bp) comprising the sequence as shown in Fig. 1 spanning the region indicated as Mitf(+) but excluding this region, for use in detecting the Mitf(-) spliced form of Mitf. It is likely that both spliced forms, i.e. both transcripts, of Mitf (+) and Mitf (-) will be present in the cells under test. However, it has been appreciated for the first time by the present inventors that the comparative expression of the transcripts alters between normal cells and cancer cells, e.g. melanocytes as compared to melanoma cells. Therefore, the detection of the different spliced forms of Mitf may be followed by comparison of the relative expressed amounts of each form, e.g the amount of mRNA/cDNA or the relative amount, e.g. ratio of expressed polypeptides.

Binding of a probe to a target nucleic acid (e.g. cDNA or mRNA) may be measured using any of a variety of techniques at the disposal of those skilled in the art. For instance, probes may be radioactively, fluorescently or enzymatically labelled. Other methods not employing labelling of probes include examination of restriction fragment length polymorphisms, amplification by PCR, Rnase cleavage and allele specific oligonucleotide probing.

Probing may employ the standard Southern or Northern blotting technique. For instance mRNA or cDNA may be extracted from cells and digested with different restriction enzymes. Restriction fragments may then be separated by electrophoresis on an agarose gel, before denaturation and transfer to a nitrocellulose filter. Labelled probe may be hybridised to the cDNA or RNA fragments on the filter and binding or intensity of

binding determined. CDNA for probing may be prepared from RNA preparations from cells.

Nucleic acid sequences derived from the sequence shown in Fig. 1 and particularly the sequence spanning the Mitf(+) sequence, are useful for (a) identifying the presence or absence of the Mitf(+) and Mitf(-) spliced forms or (b) quantifying the transcribed amounts of the Mitf (+) and Mitf (-) spliced forms in a test sample. The present invention provides a method of obtaining nucleic acid of interest, the method including hybridisation of a probe having a sequence including the Mitf (+) sequence as shown in Fig.1, variant mutant or allele thereof or a complementary sequence, to the target nucleic acid, preferably mRNA or cDNA. Hybridization is generally followed by identification and quantification of successful hybridization. In a particular preferred method, mRNA is extracted from cells under test and cDNA is produced therefrom using Reverse transcriptase PCR (RT-PCR). The produced cDNA will therefore reflect the mRNA forms of Mitf expressed in the test cell. Known techniques can then be carried out using the cDNA to determine the character and quantity of the expressed Mitf forms, for example, hybridization probes, Rnase protection etc. A further preferred method includes in situ hybridization where labelled antisense oligonucleotides of approximately 14 to 100 nucleotides, preferably 20 to 60 nucleotides, even more preferably 40 to 50 nucleotides, are contacted with the cells under test and hybridization to the mRNA transcripts are detected using the label, e.g. radioactivity where the oligonucleotides are end labelled using, for example $\alpha^{35}S$ dATP. Such oligonucleotides may also be used on Northern blots.

Further, there are provided nucleic acid molecules (oligonucleotides) for use as primers for amplification

procedures such as PCR for detecting the presence or absence of the Mitf (+) and Mitf (-) spliced forms or to determine which spliced form is predominantly present in a sample. An oligonucleotide for use in nucleic acid amplification may have about 30 bp or fewer in length. Generally specific primers are upwards of 14 nucleotides in length, but not more than 18 to 20. Those skilled in the art are well versed in the design of primers for use in processes such as PCR. Primers may be derived from the sequence shown in Fig. 1 and may, for example, be derived from the sequence indicated in the Figure. By designing primers that will amplify a region of nucleic acid including the Mitf(+) region, the presence of the two spliced forms will be indicated by the amplification of two nucleic acid molecules having different lengths, i.e. one nucleic acid molecule comprising the Mitf(+) region indicated in Fig. 1 and the other having this region missing and as a consequence being approximately 18 nucleotide bases shorter. The skilled person can determine the different sizes of the resulting amplified nucleic acid molecules by, e.g. gel electrophoresis or standard sequencing protocols. Further, the amounts of the two spliced forms can be quantified by standard methods such as autoradiography, spectrophotometry densitometry and fluorometry

The present invention also provides specific binding members comprising an antibody binding domain with specificity for one or more epitopes characteristic of Mitf(+) and Mitf(-) respectively. As the spliced variants comprise a different nucleic acid sequence, the encoded polypeptides will also differ in their amino acid sequence. This means that the encoded polypeptides will differ from each other with regard to their immunogenic properties. In other words, the two forms of encoded polypeptide will possess distinguishing epitopes as a

result of different amino acid sequence and/or as a result of different folding or conformational properties. These distinguishing immunogenic properties may be utilised in accordance with the present invention.

The specific binding members may comprise antibodies, either monoclonal or polyclonal. Alternatively they may comprise derivatives, synthetic analogues or fragments of such antibodies which retain an antibody binding domain with the specificity described above.

The inventors' provision of the sequences herein, allows one of ordinary skill in the art to make monoclonal and polyclonal antibodies which specificity for the desired polypeptide/protein by the utilization of standard procedures well known in the art. Of course, having once produced an antibody, they may be altered to produce antibody derivatives, fragments or functional equivalents (with respect to specificity) which whilst differing from the original antibody, retain an antibody binding domain or required specificity. The term "specific binding member comprising an antibody binding domain" as used herein hence covers both monoclonal and polyclonal antibodies as well as fragments, derivatives and functional equivalents thereof.

The present invention provides a specific binding member which is either (a) specific for the Mitf (+) polypeptide sequence as shown in Fig. 1 or a derivative, allele, mutant or fragment thereof; (b) specific for the Mitf(-) polypeptide sequence as shown in Fig. 1 or a derivative, allele, mutant or fragment thereof; or (c) specific for either a Mitf(+) or Mitf(-) polynucleotide sequence.

The amount of each spliced variant form, Mitf(+) or Mitf(-) may be determined by standard techniques known to the skilled person, for example, the binding members may

be labelled for detection and quantification, e.g. radioactive labelling, fluorescent labelling, enzyme labelling, or further antibody labelling.

The provision of the novel Mitf (+) and Mitf (-) polypeptides as shown in Fig. 1. enables for the first time the production of antibodies able to bind them specifically. Accordingly, a further aspect of the present invention provides an antibody able to bind specifically to a Mitf(+) or Mitf(-) polypeptides whose sequences is given in Fig. 1. Such antibodies may be specific in the sense of being able to distinguish between the polypeptides they are able to bind and other human polypeptides for which they have no or substantially no binding affinity (e.g. a binding affinity of about 1000x worse). Specific antibodies bind an epitope on the molecule which is either not present or is not accessible on other molecules. Where a panel of antibodies are used, e.g. different antibodies which are independently specific for each spliced variant form, different reporting labels may be employed for each antibody so that binding of each can be observed and quantified.

As described above, the present inventors have determined that differential expression of the two spliced variant forms of Mitf (-) and Mitf (+) occur in tumour cell as opposed to normal cells. In particular, the inventors have shown the Mitf (-) form is predominantly expressed in tumour cells. As a result of this, detection and quantification of the differential expression of the two spliced variants provides a useful diagnostic tool for determining the presence, type and duration of tumours, for example, melanoma.

Methods for determining the concentration of analytes in biological samples from individuals are well known in the art and can be employed in the context of

the present invention to determine whether an individual has an elevated level of Mitf (-) expression as compared to the Mitf (+) expression, and so has or is at risk from cancer. The purpose of such analysis may be used for diagnosis or prognosis to assist a physician in determining the severity or likely course of the cancer and/or to optimise treatment of it.

Preferred diagnostic methods rely on the detection and quantification of the two spliced variant forms in biological samples such as tissue cells, for example, naevi cells, primary or secondary melanomas, mast cells or osteoclasts and other cell types expressing Mitf mRNA.

Assay methods for use in such diagnosis utilise the binding members as described above. Conveniently, where the binding members are nucleic acid probes or antibody binding regions, they may be immobilised on a solid support, e.g. at a defined location, to make it easy to manipulate during the assay.

The biological sample under test is generally contacted with the binding member under appropriate conditions so that the Mitf spliced variant forms can bind to the member. The occupancy of the binding sites of the binding members can then be determined using a developing agent or agents. Typically the developing agents are labelled (e.g. with radioactive fluorescent or enzyme labels) so that they can be detected using techniques well known in the art. Thus, radioactive labels can be detected using a scintillation counter or other radiation counting device, fluorescent labels using a laser and confocal microscope, and enzyme labels by the action of an enzyme label on a substrate, typically to produce a colour change. The developing agent can be used in a competitive method in which the developing agent competes with the spliced variant forms for occupied binding sites of the binding label, or non-competitive

method, in which the labelled developing agent binds the variant spliced forms bound by the binding member or to occupied binding sites. Both methods provide an indication of the number of the binding sites occupied by the first spliced variant form, and hence the concentration of the form in the sample, e.g. by comparison with a previous assay for the second variant form.

Thus, the present invention provides a method of diagnosing a cancer or risk of a cancer, for example, melanoma, in a patient comprising determining the level of expression of the alternative spliced variant forms mitf(+) and Mitf(-) in a biological sample obtained from a patient.

There is also an increasing tendency in the diagnostic field towards miniaturisation of assays as described above, e.g. making use of binding agents (such as antibodies or nucleic acid sequences) immobilised in small, discrete location (microspots) and/or as arrays on solid supports or on diagnostic chips. These approaches can be particularly valuable as they can provide great sensitivity (particularly through the use of fluorescent labelled reagents), require only very small amounts of biological sample from individuals being tested and allow a variety of separate assays can be carried out simultaneously. Examples of techniques enabling this miniaturised technology are provided in WO84/01031, WO88/01058, WO89/01157, WO93/88472, WO95//18376, WO95/18377, WO95/24649 and EP-A-0373203. Thus in a further aspect, the present invention provides a kit comprising a support or diagnostic chip having immobilised thereon one or more binding members capable of specifically binding either of the spliced variant forms Mitf(+) and Mitf(-), optionally in conjunction with other reagents (such as labelled developing reagents)

needed to carry out an assay.

The present inventors have also found that it is possible to switch expression from the Mitf (+) form to the Mitf (-) form of Mitf mRNA in cells by ectopic expression of a constitutively active mitogen-activated kinase kinase (MAPKK or MEK). Therefore, the present invention also provides a method for screening for candidate compounds capable of modifying expression of Mitf mRNA transcripts, said method comprising exposing cells capable of expressing Mitf mRNA to candidate compounds; detecting expression of Mitf (+) and Mitf (-) forms of Mitf mRNA; and selecting those compounds which modify the ratio of Mitf(+) expression to Mitf (-) expression. A comparison step may in included in this method whereby the ratio of Mitf(-) to Mitf (+) expression is compared to a control step in which the candidate compound has been omitted.

There is further provided a method for screening for compounds capable of inhibiting the relative increased expression of the Mitf (-) transcript as compared to the Mitf (+) transcript, said method comprising contacting a cell in an environment such that it is capable of expressing Mitf mRNA with a candidate compound and detecting the relative expression of Mitf (+) and Mitf (-) forms of Mitf mRNA; and comparing said relative expression with that in a control cell from the same environment in order to determine the inhibition capabilities of the candidate compound. Preferably the cells are melanocytes and the candidate compounds have the potential ability to inhibit the MAPkinase pathway directly or indirectly by, for example, inhibition of receptor tyrosine kinase function or RAS or other steps in the MAPkinase signalling cascade. Such a method is useful in determining compounds capable of preventing the switch to increased expression of the Mitf (-) form as

opposed to the Mitf (+) form. Such determined compounds may then be utilised in the treatment of cancer cells associated with the increased expression of the Mitf (-) transcript.

The present invention also provide a cell system model for use in testing or screening for candidate compounds capable of influencing the relative expression of the Mitf (-) form of Mitf mRNA as compared to the Mitf (+) form of Mitf mRNA, i.e screening for compounds capable of altering the ratio of the Mitf (+) and Mitf (-) forms as compared to a control system absent of said candidate compound. Such a system may further be used to monitor the progress of a cancer and/or the success of any therapeutics. Such candidate compounds may be of use as therapeutics.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

Brief Description of the Drawings

In the Figures:

Figure 1: DNA and polypeptide sequences corresponding to human (hsMitfna) or mouse (mmMitfna) Mitf. Sequences indicated in bold type correspond to the 6 amino acids uniquely present in the Mitf(+) protein. Sequences underlined indicate bases uniquely present in the Mitf(+) cDNA. The locations of the primers used for the PCR reactions are indicated.

Figure 2: A) RT-PCR using RNA derived from the indicated cell lines (melan-a, mouse melanocyte; B16, relatively differentiated mouse melanoma; MM96, human melanoma). The primers used for the PCR reaction are

indicated on the attached Mitf sequence in Figure 1.

Figure 2: B) The melan-a cell line was infected with a retrovirus expressing a 6xHIS tagged version of the constitutively activated form of the MAPkinase kinase MEK (MEK.EE) and two clonal cell lines (MEK1 and MEK2) were isolated. Constitutively active MEK expression was detected using an antibody directed against the 6xHIS tag. MEK1 express a low level of constitutively active MEK and MEK2 at higher level.

Figure 2: C) RT PCR using the same primers as in A) and RNA derived from the indicated cell lines. An Mitf(-) cDNA was used as a control. Little or no Mitf(+) mRNA is detected in the MEK1 and MEK2 cell lines.

Figure 2: D) Control: PCR using specific Mitf primers and a mixture of the Mitf(+) and Mitf(-) cDNAs as a control for the size of the PCR products expected. M: size markers derived from 100bp ladder (BRL) showing positions of 200 and 300bp markers. Dx3, HMB2, MeWo, VUP, A375P and A375M are all human melanoma cell lines provided by Prof. Ian Hart. St Thomas's Hospital London.

Detailed description

Using RNA derived from the melanocyte cell line melan-a together with reverse transcription coupled with the polymerase chain reaction (RT-PCR) using primers corresponding to sequences flanking the differential splice, it is possible to distinguish PCR products corresponding to cDNAs derived from the Mitf(+) and Mitf(-) mRNA. In the mouse melanocyte cell line melan-a, and in the relatively differentiated mouse melanoma cell line B16, the predominant form of Mitf mRNA expressed is the Mitf(+) form (Figure 2A).

In contrast, in two human melanoma cell lines MM96 and 501mel, RT-PCR reveals that the Mitf(-) form of Mitf mRNA is the predominant form present (Figure 2A)

The present inventors have also found that it is possible to switch expression from the (+) form to the (-) form of Mitf in the melanocyte melan-a cell line by the ectopic expression of a constitutively active mitogen-activated kinase (MAPKK or MEK) (Figure 2B and C). Since Mek activates MAPkinases such as ERK2, it would appear that constitutive activation of the MAPkinase signalling pathway leading to activation of MEK and ERKs results in cells expressing the Mitf (-) form in preference to the Mitf (+) form.

The preferential expression of the Mitf (-) form of Mitf mRNA in melanoma cells might be accounted for by constitutive signalling by the MAPkinase pathway, resulting either from the constitutive activation of receptor tyrosine kinases, a characteristic of melanomas, or other events leading to constitutive activation of the MAPkinase signalling cascade eg, activating mutations of Ras (reviewed in Chin et al., 1998; Genes Dev. 12, 3467-3481).

The expression of differentially spliced forms of Mitf mRNA or protein may be detected by

(i) RT-PCR,

(ii) by fluorescent in situ hybridisation using a probe hybridizing specifically to either the (+) or (-) forms of Mitf mRNA,

(iii) by using a specific antibody directed against epitopes uniquely present in either the (-) or (+) form of Mitf protein.

These reagents may be used to determine the relative proportions of Mitf (-) or (+) forms of mRNA or protein present in cell lines, or in primary tissue derived from, for example, naevi or primary or secondary melanomas. The ratio of Mitf (-) and Mitf (+) forms detected are considered to be indicative of the state of activation of the MAPkinase or other signalling cascades and as such

can be used as a prognostic or diagnostic marker for melanoma progression or as a means to monitor changes in melanomas following treatment.

All potential applications for melanocytes and melanomas should also be applicable to other cells types in which Mitf is expressed eg. mast cells or osteoclasts.

An example of a cell system for determining the relative expression of the two Mitf splice variants is described as follows.

The untransformed melanocyte-cell line (Bennett, D.C., et al. Int J. Cancer 39:414-418) was infected with a retrovirus (pBabePuro) containing a drug resistance marker and expressing a dominant positive form of the MAPKK containing the S217E, S221E mutation, with a 6xHis tag at the C-terminus. The WT MAPKK has a Genbank accession No. Z30163, while mutant derivative of MAPKK has been described previously (Cowley, S., et al (1994), Cell, 77, 841-852)and the retroviral expression vector was provided by Prof. Chris Marshall (Institute for Cancer Research London). The infected melan-a cells were placed in selection medium containing puromycin to permit the growth of retrovirally infected cells and clonal cell lines established using standard cell cloning techniques. The two derivative cell lines, MEK1 and MEK2, were shown to express low or high levels of constitutively active MEK by western blotting using an anti-His tag antibody (see Fig 2). Whole cell RNA from all cell lines was isolated using standard techniques and a reverse transcription reaction performed using random primers and AMV reverse transcriptase.

The relative proportions of Mitf(-) and Mitf(+) mRNAs was determined following PCR using the primers indicated in Fig. 1 followed by agarose gel electrophoresis and visualisation under UV light after staining with ethidium bromide.

Figure 2D illustrates tests to determine the ratio between the Mitf(-) and Mitf(+) forms in various human melanoma cell lines. In brief, total cytoplasmic RNA was prepared from the indicated cell lines and subjected to RT PCR using specific Mitf primers (see Fig. 1). The PCR products obtained were resolved by agarose gel electrophoresis before visualisation by staining with ethidium bromide and photography under UV light.

The differences in ratio between the Mitf(-) and Mitf(+) forms are readily observed. Most notably, the difference is seen in the A375P and A375M lines. A375P has been described as a human low metastatic cell line and A375M as a high metastatic variant of A375P (see for example Hendrix, M.J. et al (1987): A simple quantitative assay for studying the invasive potential of high and low human metastatic variants. Cancer Lett. 38, 137-147. and references therein).

Claims

1. A method of determining the predominance of either of the splice variants Mitf(-) and Mitf(+) in a biological sample, said method comprising the steps of independently detecting Mitf(-) and Mitf(+) followed by comparing the amounts of said spliced variants in order to determine which splice variant is predominant in said sample.

2. A method according to claim 1 wherein said splice variants are independently detected on the basis of their size difference.

3. A method according to claim 1 or claim 2 wherein said splice variants are in the form of mRNA and said method further comprises amplifying said splice variants prior to detection using RT-PCR to produce cDNA corresponding to either Mitf(-) or Mitf(+).

4. A method according to claim 3 wherein said RT-PCR uses nucleic acid primers designed from the sequence shown in Figure 1.

5. A method according to claim 4 wherein said primers correspond to primer 1 and primer 2 as identified in Figure 1.

6. A method according to any one of claims 3 to 5 wherein said cDNA corresponding to either Mitf(-) or Mitf(+) is independently detected on the basis of size by electrophoresis.

7. A method according to claim 1 wherein said splice variants are independently detected using specific binding members capable of specifically binding to either Mitf(-) or Mitf(+).

8. A method according to claim 7 wherein said specific binding members are nucleic acid probes.

9. A method according to claim 7 wherein said sliced variants are in the form of polypeptides and said specific binding members are antibodies.

10. A method according to claim 8 or claim 9 wherein said nucleic acid probes or said antibodies are labelled to assist detection.

11. A nucleic acid molecule having between 20bp and 100bp comprising the sequence as shown in Figure 1 spanning the region indicated as Mitf(+) but excluding this region, for use in detecting the Mitf(-) form of Mitf.

12. A nucleic acid molecular having between 20bp and 100bp comprising the sequence as shown in figure 1 spanning the region indicated as Mitf(+) and including this region, for use in detecting the Mitf(-) form of Mitf.

13. A nucleic acid molecule for use as a primer for amplification procedure such as PCR for detecting the presence or absence or the Mitf(-) and Mitf(+) spliced forms or to determine which spliced form is predominantly present in a sample, said nucleic acid molecule comprising a sequence designated primer 1 or primer 2 as shown in Figure 1.

14. A nucleic acid molecule according to claim 13 having less than 30bp.

15. A specific binding member which is either (a) specific for Mitf(+) polypeptide sequence as shown in Figure 1 or a derivative, allele, mutant or fragment

thereof; (b) specific for the Mitf(-) polypeptide sequence as shown in Fig. 1 or a derivative, allele, mutant or fragment thereof; or (c) specific for either a Mitf(-) or Mitf(+) polynucleotide sequence.

16. A specific binding member according to claim 15 comprising an antibody domain specific for one or more epitopes characteristic of Mitf(+) or Mitf(-) respectively.

17. A specific binding member according to claim 15 which is labelled to aid detection and quantification.

18. A specific binding member according to any one of claims 15 to 17 immobilised on a support or diagnostic chip.

19. Use of a specific binding member according to any one of claims 15 to 18 for diagnosing a disease state or risk of said disease state in a patient.

20. Use of a specific binding member according to any one of claims 15 to 17 in the preparation of a diagnostic or prognostic tool for determining a disease state or risk of a disease state in a patient.

21. A method of diagnosing a disease state or risk of a disease state in a patient comprising determining the level of expression of the alternative spliced variant forms mitf(+) and Mitf(-) in a biological sample obtained from a patient.

22. A method according to claim 21 wherein the disease state is cancer.

23. A method according to claim 22 wherein the cancer is melanoma.

24. A kit for use in determining the predominance of Mitf(+) or Mitf(-) in a biological sample in a method according to any one of claims 1 to 10, said kit comprising nucleic acid primers according to claim 12 or claim 14.

25. A kit according to claim 24 further comprising a support or diagnostic chip having immobilised thereon one or more specific binding members capable of specifically binding either of the spliced variant forms Mitf(+) and Mitf(-).

26. A method of screening for candidate compounds capable of affecting expression of Mitf mRNA transcripts, said method comprising exposing cells capable of expressing Mitf mRNA to candidate compounds; detecting expression of Mitf(+) and Mitf(-) forms of Mitf mRNA; and selecting those candidate compounds which affect the ratio of Mitf(+) expression to Mitf(-) expression.

27. A method of screening for compounds capable of inhibiting the relative increased expression of the Mitf(-) transcript as compared to the Mitf(+) transcript, said method comprising contacting a cell in an environment such that it is capable of expressing Mitf mRNA with a candidate compound and detecting the relative expression of Mitf(-) and Mitf(+) forms of Mitf mRNA; and comparing said relative levels of expression with that in a control cell from the same environment in order to determine the inhibition capabilities of the candidate compounds.

28. A method according to claim 27 wherein the cell is a melanocyte.

29. A method according to claim 27 or claim 28 wherein the step of detecting the relative expression of Mitf(-)

and Mitf(+) forms is carried out in accordance with the method of claim 1.

30. A method according to any one of claims 27 to 29 wherein said candidate compounds are initially selected owing to their potential ability to inhibit the MAPkinase pathway.

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Figure 1

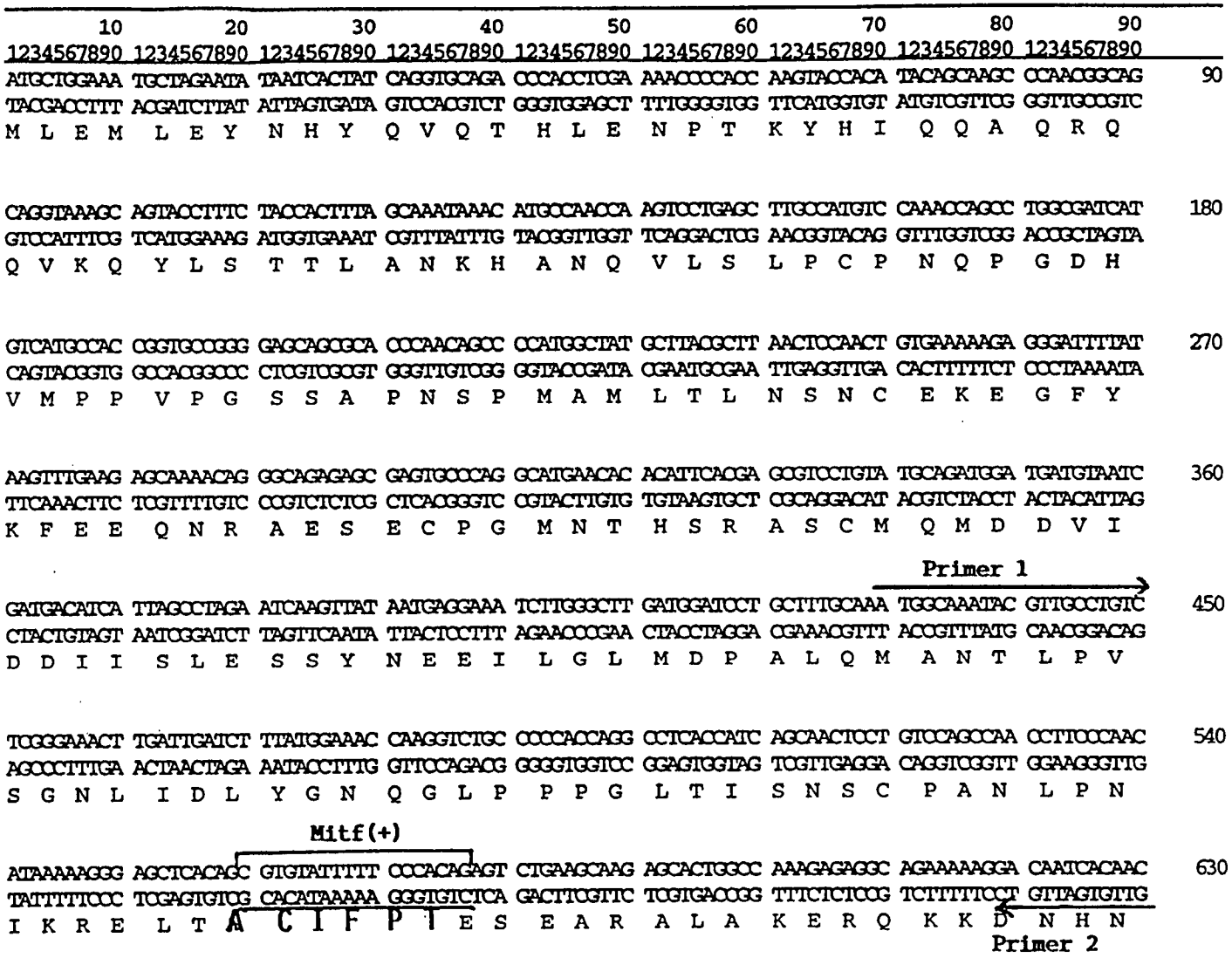

Figure 1 continued

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CTGATTGAAC GAAGAAGAAG ATTTAACATA AATGACCCCA TTAAAGACT AGGTACTTTG ATTCCCAAGT CAAATGATCC AGACATGCGC      720
GACTAACTTG CTTCTTCTTC TAAATTGTAT TTACTGGGGT AATTTCTTGA TCCATGAAAC TAAGGTTCA GTTTACTAGG TCTGTACGCG
L  I  E  R  R  R  R  F  N  I  N  D  R  I  K  E  L  G  T  L  I  P  K  S  N  D  P  D  M  R

TGGAACAAGG GAACCATCTT AAAAGCATCC GTGGACTATA TCCGAAAGTT GCAACGAGAA CAGCAACCCG CAAAGAACT TGAAAACCGA      810
ACCTTGTTCC CTTGGTAGAA TTTTCGTAGG CACCTGATAT AGGCTTTCAA CGTTGCTCTT GTCGTTGGGC GTTTTCTTGA ACTTTTGGCT
W  N  K  G  T  I  L  K  A  S  V  D  Y  I  R  K  L  Q  R  E  Q  Q  R  A  K  E  L  E  N  R

CAGAAGAAAC TGGAGCAGCC CAACCGGCAT TTGTTCCTCA GAATACAGGA ACTTGAAATG CAGGCTCGAG CTCATGGACT TTCCCTTATT      900
GTCTTCTTTG ACCTCGTCGG GTTGGCCGTA AACAACGAGT CTTATGTCCT TGAACTTTAC GTCCGAGCTC GAGTACCTGA AAGGGAATAA
Q  K  K  L  E  H  A  N  R  H  L  L  L  R  I  Q  E  L  E  M  Q  A  R  A  H  G  L  S  L  I

CCATCCAGGG GTCTCTGCTC TCCAGATTTG GTGAATCGGA TCATCAGCA AGAACCCGTT CTTGAGAACT GCAGCCAAGA CCTCCTTCAG      990
GGTAGGTCCC CAGAGACGAG AGGTCTAAAC CACTTAGCCT AGTAGTTCGT TCTTGGCCAA GAACTCTTGA CGTCGGTTCT GGAGGAAGTC
P  S  T  G  L  C  S  P  D  L  V  N  R  I  I  K  Q  E  P  V  L  E  N  C  S  Q  D  L  L  Q

CATCATGCAG ACCTAACCTG TACAACAACT CTCGATCTCA CGGATGGCAC CATCACCTTC AACACAACC TCGGAACTGG GACTGAGCCC      1080
GTAGTACGTC TGGATTGGAC ATGTTGTTGA GAGCTAGAGT GCCTACCGTG GTAGTGGAAG TTGTTGTTGG AGCCTTGACC CTGACTCGGG
H  H  A  D  L  T  C  T  T  T  L  D  L  T  D  G  T  I  T  F  N  N  N  L  G  T  G  T  E  A

AACCAAGCCT ATAGTGTCCC CACAAAAATG GGATCCAAAC TGGAAGACAT CCTGATGGAC GACACCCTTT CTCCCGTCGG TGTCACTGAT      1170
TTGGTTCGGA TATCACAGGG GTGTTTTTAC CCTAGGTTTG ACCTCTGTA CGACTACCTG CTGTGGGAAA GAGGGCAGCC ACAGTGACTA
N  Q  A  Y  S  V  P  T  K  M  G  S  K  L  E  D  I  L  M  D  D  T  L  S  P  V  G  V  T  D

CCACTCCTTT CCTCAGTGTC CCCCCGAGCT TCCAAAACAA GCAGCCGGAG GAGCAGTATG AGCATGGAAG AGACGGAGCA CACTTGTTAG      1260
GGTGAGGAAA GGAGTCACAG GGGGCCTCGA AGGTTTGTTT CGTCGGCCTC CTCGTCATAC TCGTACCTTC TCTGCCTCGT GTGAACAATC
P  L  L  S  S  V  S  P  G  A  S  K  T  S  S  R  R  S  S  M  S  M  E  E  T  E  H  T  C  .
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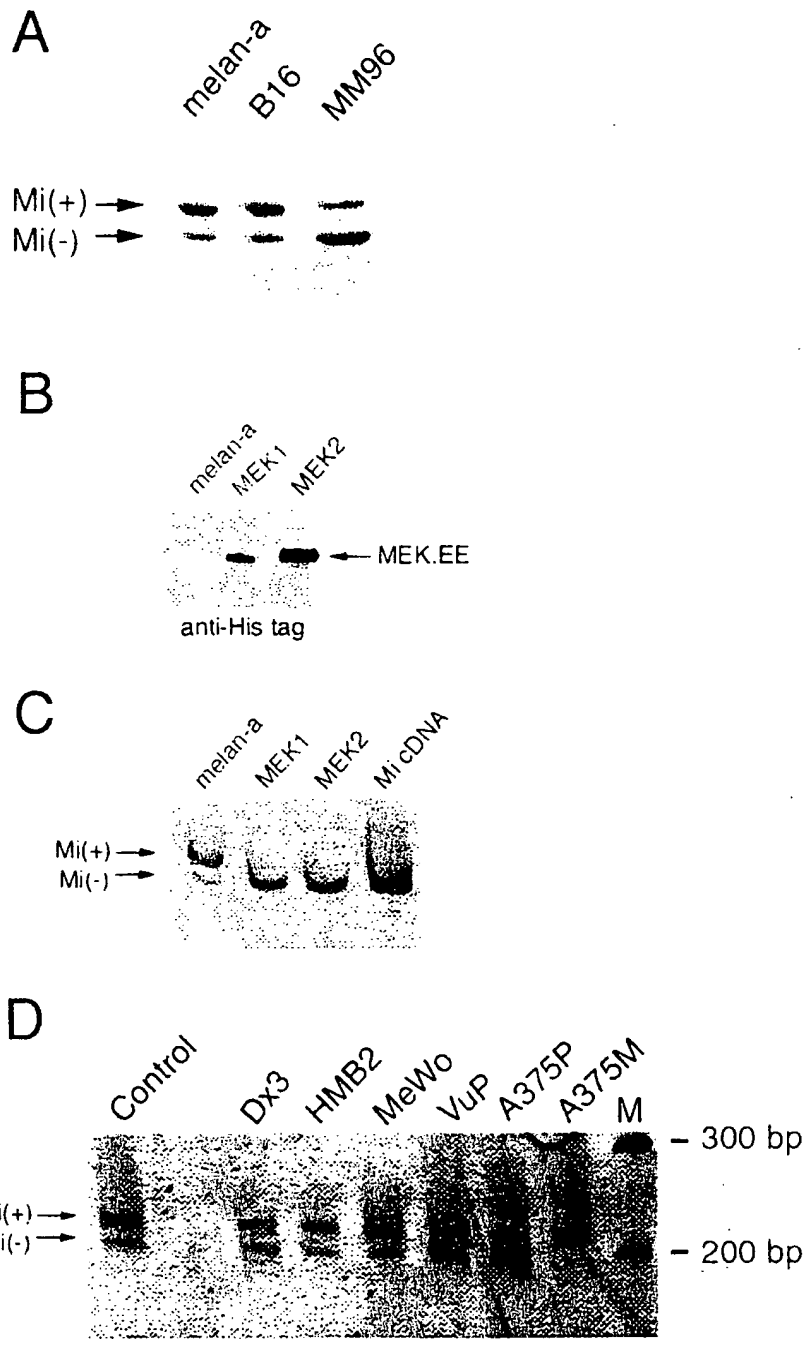

Figure 2

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INTERNATIONAL SEARCH REPORT

Interr. nal Application No
PCT/GB 00/00313

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YASUMOTO K-I ET AL: "A big gene linked to small eyes encodes multiple Mitf isoforms: Many promoters make light work" PIGMENT CELL RESEARCH, vol. 11 , no. 6, December 1998 (1998-12), page 329-336 XP000909094 page 332, paragraph 5 - paragraph 6	1-8, 10-14, 24-29
Y	STEINGRIMSSON E ET AL: "Molecular basis of mouse microphthalmia (MI) mutations helps explain the developmental and phenotypic consequences" NATURE GENETICS, vol. 8, no. 3, November 1994 (1994-11), pages 256-63, XP000909096 cited in the application the whole document	1-8, 10-14, 24-29

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 May 2000	22/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

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INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/GB 00/00313

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	TASSABEHJI M ET AL: "Waardenburg syndrome type 2 caused by mutations in the human microphthalmia (MITF) gene" NATURE GENETICS, vol. 8, no. 3, November 1994 (1994-11), pages 251-5, XP000909097 the whole document	1-8, 10-14, 24-29
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A	BERTOLOTTO C ET AL: "Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes" JOURNAL OF CELL BIOLOGY, vol. 142, no. 3, August 1998 (1998-08), pages 827-35, XP000908972 page 832, paragraph 2	7,9, 15-17
A	WO 97 15688 A (UNIV WASHINGTON ;UNIV SOUTH FLORIDA (US); HARDY JOHN A (US); GOATE) 1 May 1997 (1997-05-01) page 4, line 8 - line 20	1-29

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INTERNATIONAL SEARCH REPORT

information on patent family members

Inter. nal Application No
PCT/GB 00/00313

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9715688 A	01-05-1997	AU 7521696 A	15-05-1997
		EP 0859862 A	26-08-1998
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		ZA 9608899 A	23-07-1998